

Seed specific expression of perilla γ -tocopherol methyltransferase gene increases α -tocopherol content in transgenic perilla (*Perilla frutescens*)

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Received: 21 May 2007 / Accepted: 16 September 2007 / Published online: 17 October 2007
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Abstract Increasing vitamin E activity in economically important oil crops such as perilla will enhance the nutritional value of these crops. Perilla (*Perilla frutescens* Britt) represents an important oil crop in Asian countries, including Korea. Using *Agrobacterium*-mediated transformation, we have engineered perilla with the γ -tocopherol methyltransferase (γ -TMT) gene under the control of seed-specific vicillin promoter. Molecular characterization including PCR, Southern and Northern blots confirmed that the γ -TMT transgene was successfully inherited to and expressed in the progeny plants. The γ -TMT transgene was specifically expressed in immature seeds of transgenic plants, leading to efficient conversion of γ -tocopherol to α -tocopherol and dramatic increase in seed α -tocopherol content, as detected by

high performance liquid chromatography analysis. We also showed that such a high α -tocopherol content phenotype was transmitted to the progeny plants. In addition, there was no significant change in fatty acid composition in transgenic seeds as compared with untransformed control Yeupsil cultivar, suggesting the lack of interplay between the fatty acid and tocopherol biosynthesis pathways. This was the first report on over expression of the γ -TMT gene in transgenic perilla displaying desirable high α -tocopherol content phenotype. Since α -tocopherol has the highest vitamin E activity, the transgenic perilla with high α -tocopherol content in seeds developed in this study will benefit both human and animal health.

Keywords Perilla · α -tocopherol · γ -tocopherol · *Agrobacterium*-mediated transformation · Seed-specific expression

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Abbreviations

γ -TMT γ -tocopherol methyltransferase
bar gene Bialaphos resistance gene
HPLC High performance liquid chromatography
GC Gas chromatography

Introduction

Vitamin E is an essential nutritional element for both humans and animals and therefore must be

adequately provided by foods or other supplements. Vitamin E is a collective term for unsaturated α -, β -, γ - and δ -tocopherols and tocotrienols (Eitenmiller 1997). Alfa-tocopherol is considered to be the most important form of vitamin E for human or animal health (Traber and Sies 1996) since α -tocopherol has the highest vitamin E activity among tocopherols. The relative activity or potency of β -, γ - and δ -tocopherols to α -tocopherol is estimated to be 0.5, 0.1 and 0.03, respectively, in human (Kamal-Eldin and Appelqvist 1996). The major sources of vitamin E for human or animal consumption are from plants, but the concentration of vitamin E varies depending on plant species.

Of various plant species, oil crop plants generally contain high levels of tocopherols in their seeds. However, most common oilseeds contain relatively higher levels of γ -tocopherol, a precursor of α -tocopherol than α -tocopherol itself (Shintani and DellaPenna 1998; Grusak 1999). Therefore, it would be desirable for more efficient intake by human or animals if γ -tocopherol could be metabolically converted to α -tocopherol (Shintani and DellaPenna 1998). Plant transgenic technology has been quite successful in introducing and subsequently changing the tocopherol composition by overexpressing the γ -TMT gene to convert the precursor γ -tocopherol to α -tocopherol (Shintani and DellaPenna 1998; Kim et al. 2000; Cho et al. 2005). Shintani and DellaPenna (1998) reported that the seeds of transgenic *Arabidopsis* overexpressing γ -TMT driven by the carrot DC3 seed-specific promoter showed an 80-fold increase in α -tocopherol levels as compared with the wild-type control. It was also reported that many useful crops with high α -tocopherol content could be developed by introducing this gene. Cho et al. (2005) and Kim et al. (2000) developed transgenic lettuce (*Lactuca sativa* L.) with high level of α -tocopherol by overexpressing a cDNA encoding the γ -TMT gene from *Arabidopsis thaliana*. Recently it was shown that transgenic soybean (Tavva et al. 2007) and transgenic *Brassica juncea* (Yusuf and Sarin 2007) with increased α -tocopherol content were developed by expressing a perilla γ -TMT gene.

Gamma-TMT genes encoding γ -tocopherol methyltransferase have been previously cloned from different plant species such as *Arabidopsis thaliana* (Shintani and DellaPenna 1998) and *Perilla frutescens* (Kim et al. 2002). Therefore, it would now be possible

to convert γ -tocopherol to α -tocopherol by overexpression of a γ -TMT gene under the control of a seed-specific promoter in the seeds of engineered crops.

Perilla is cultivated as an oil crop, medicinal crop or as a vegetable in Asian countries such as Korea, Japan, northeast China and Nepal (Honda et al. 1990). Oil content in perilla seeds is about 46% (w/w) (Park et al. 2000). Gamma-tocopherol is the most abundant tocopherol in perilla seed like those of other oil crops such as soybean, rapeseed, and corn (Grusak 1999). Therefore, it would be advantageous to increase the α -tocopherol content in perilla by introducing a γ -TMT gene into perilla thereby enhancing conversion from γ -tocopherol to α -tocopherol. We have previously reported the establishment of a regeneration system (Lee et al. 2003), *Agrobacterium*-mediated transformation system of perilla and the development of transgenic perilla with perilla γ -TMT gene under the control of seed-specific vicillin promoter (Lee et al. 2005).

Therefore, this study was undertaken to analyze transgenic perilla for the expression of γ -TMT gene in a seed-specific manner. Here we show that the α -tocopherol content increased dramatically in the seeds of transgenic perilla. Furthermore, it is also demonstrated that both the transgenes and the high α -tocopherol content phenotype were stably inherited to progeny plants.

Materials and methods

Plant materials

Four independent transgenic T_0 plant lines (P1, P2, P3, P4) were obtained by *Agrobacterium*-mediated transformation using vector pBK I (Lee et al. 2005) carrying the γ -TMT gene isolated from perilla (Kim et al. 2002) under the control of the vicillin promoter. To advance generation, six T_1 progeny plants (P1-1, P1-2, P1-3, P2-1, P2-2, and P2-3) derived from two T_0 lines (P1, P2) were obtained by self pollination. To further advance generation, six T_2 progeny plants (P1-2-1, P1-2-2, P1-2-3, P1-3-1, P1-3-2, and P1-3-3) derived from two T_1 plants (P1-2 and P1-3) were obtained by self pollination. The Korean elite variety, Yeupsil (Lee et al. 1989) was used as an untransformed control. The pBK I carries the *bar* gene as a plant selectable marker under the control of

cauliflower mosaic virus (CaMV) 35S promoter and the perilla γ -TMT gene (Kim et al. 2002) driven by the seed-specific vicillin promoter (Higgins et al. 1988). All transgenic plants were grown in plastic pots filled with soil mixture (soil:sand:vermiculite = 1:1:1) in the greenhouse. The photoperiod was adjusted to 16/8 h (light/dark) and the temperature was maintained at $25 \pm 3^\circ\text{C}$ for two months. Thereafter, the photoperiod was changed to 14/10 h (light/dark) to accelerate flowering. Leaves and immature seeds were collected from both transgenic perilla and untransformed control, cv. Yeupsil about 10 days after flowering and used for Southern and Northern blot analyses.

Molecular characterization of transgene in perilla

Transgenic plants and their progeny were first screened by leaf-painting assay by applying 0.5% BASTA onto a fully-expanded leaf of each plant a month after planting. To obtain sufficient seeds, three T_1 plants (P1-1, P1-2, and P1-3) were assayed by leaf-painting at the seed mature stage.

To confirm transgene inheritance and segregation, genomic DNA was isolated from young leaf tissues of the transgenic plants as well as wild type Yeupsil using the CTAB method (Murray and Thompson 1980) with minor modification. To detect the γ -TMT transgene by PCR, a specific primer pair was designed to specifically amplify the γ -TMT transgene rather than endogenous γ -TMT in perilla. The primer sequences are: forward 5'-CATTACAT GCCGAA AACCTGCATCTTAA-3' (from γ -TMT transgene) and reverse 5'-TGAACGTTATTAGTTCCGCCGCTC GGTG -3' [from the octopine synthase (OCS) terminator of pBK I]. The PCR reaction mixture contained 100 ng of genomic DNA or 100 pg of plasmid DNA, 0.5 μM of each primer, 100 μM dNTP mixture, 1 \times Taq DNA polymerase reaction buffer and 1 unit Taq DNA polymerase (Promega, USA) in a 50 μl reaction volume. PCR amplification was performed as follows: one cycle at 96°C for 30 s. for hot start, 30 cycles of reactions at 96°C for 30 s. for denaturation, 60°C for 30 s. for annealing, 72°C for 30 s. for extension, and 72°C for 7 min for final extension. Amplified products were electrophoresed on 0.9% agarose-EtBr gel and visualized under UV light.

To further confirm transgene inheritance in the T_2 progeny plants, Southern blot analysis was conducted following a procedure modified from Sambrook and Russel (2001). Ten μg of genomic DNA was digested with *EcoRI*, separated on 1% agarose gel by electrophoresis, and then transferred to Hybond-N+ nylon membrane (Amersham, USA) by capillary transfer for 16 h. The transferred DNA was fixed to the membrane by UV cross-linking. A 0.7 kb amplified PCR product from the γ -TMT and OCS gene was labeled using DIG high prime DNA labeling and detection starter kit II (Roche Applied Science) and used as a probe. To confirm the tissue-specific expression of the transformed γ -TMT gene, total RNA was extracted from young leaves or immature seeds about 10 days after flowering using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) following the manufacturer's instructions. Fifteen μg of each RNA sample was electrophoresed in 1% (w/v) agarose-formaldehyde gel, transferred to a Hybond-N+ nylon membrane (Amersham, USA), and fixed by UV cross-linking. The RNA was hybridized with the same DNA probe for Southern blot analysis but labeled with [^{32}P]-dCTP using a random primer labeling kit (Stratagene, USA). Conditions for the hybridization were the same as described for the Southern blot.

Analysis of tocopherols, total lipids and fatty acids in transgenic perilla seeds

Sample preparation

Seeds were harvested from transgenic plants and Yeupsil and dried at 25°C for 1 month. The seeds were crushed to coarse grains with a mortar and pestle and then milled to about 100 mesh by Brabender Test Mill (Brabender Instruments, Inc., Duisburg, Germany) and used for the analyses of tocopherols, total lipids and fatty acids.

Analysis of tocopherols

Tocopherols were analyzed by the modified method of Gimeno et al. (2000) and Kamal-Eldin et al. (2000). Two gram of milled sample was mixed with 5.0 ml of hexane in a 15 ml test tube by vortexing

and incubated for 3 h at room temperature. Thereafter, the hexane extract was filtered through Sep-Pak NH₂ cartridge and then dried at 60°C with a dry-bath (Thermolyne, USA) for 30 min. After drying, each sample was diluted in 2.0 ml of hexane, and 20 µl of the diluted sample was directly injected for the high performance liquid chromatography (HPLC) analysis. The standard α -, γ - and δ -tocopherols were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA) and used along with the samples to be analyzed. The HPLC analysis was performed on the Shimadzu system (Shimadzu Co., Kyoto, Japan) equipped with SPD-7AV UV detector, LC-7A pump, SCL-6B system controller, and C-R6A integrator. The column was Supelcosil LC-NH₂ (25 cm × 4.6 mm, 5 µm) and operated at a temperature of 30°C. A mixture of hexane: ethyl acetate (70:30, v/v) was used as a mobile phase at a flow rate of 1.0 ml/min, and detection was accomplished by monitoring at 292 nm.

Analysis of total lipids

Total lipid content of perilla seeds was measured by Soxtherm automatic system (Gerhardt, Germany). The extraction beaker was filled with a few boiling stones and then dried at 105°C. Five g of the homogenized sample was placed into an extraction thimble and 140 ml of *n*-hexane was added. After boiling for 30 min at 180°C, extraction was performed for 80 min with solvent reduction for five times. After extraction, the beakers were dried at 105°C for 1 h, then cooled down to room temperature in a desiccator and weighed.

Analysis of fatty acids

The fatty acids were analyzed using the method of Rafael and Mancha (1993). About 0.5 g of sample was heated together with a reagent containing methanol: heptane: benzene: 2,2-dimethoxypropane: H₂SO₄ = 37: 36: 20: 5: 2 (v/v). The simultaneous digestion and lipid transmethylation took place in a single phase at 80°C. After cooling at room temperature, the upper phases containing the fatty acid methyl ester (FAME) were used for the capillary gas chromatography (GC) analysis. The GC analysis was

performed on the Agilent 6890 system (HP Co., Wilmington, DE, USA) equipped with a flame ionization detector (FID) by using a HP-Innowax capillary column (30 m × 0.25 mm × 0.25 µm film, cross-linked polyethylene glycol). The initial temperature of 150°C was increased to the final temperature of 280°C at the rate of 4°C/min. Nitrogen gas was used as carrier at a flow rate of 10 ml/min. During the analysis, the temperatures of inlet and detector were maintained at 250°C and 300°C, respectively. The standard FAME Mix (C14-C22) was purchased from Supelco (Bellefonte, PA, USA).

Results

Confirmation of transgene inheritance and segregation

Transgenic perilla plants were first confirmed by the application of 0.5% BASTA brushing one leaf per plant a month after transplanting. Putative T₀ transgenic perilla plants and their progeny plants were assayed. The results showed that all the plants tested except for a T₁ plant (P1-1) were tolerant to the herbicide (Fig. 1a). This herbicide assay agreed well with PCR confirmation of the transgene (Fig. 1b). The Southern blot showed the presence of the transgene in three T₂ plants derived from the same T₂ plant (P1-2), also confirming a stable inheritance of the transgene into the progeny generation (Fig. 1c).

Transcript analysis of tissue specific expression of transformed γ -TMT gene

Northern blot analysis was performed to examine whether the γ -TMT transgene was expressed specifically within the seeds, since the transgene expression was controlled by the seed specific vicillin promoter. Total RNA isolated from either leaves or immature seed samples was used for Northern blot analysis. The result showed that the γ -TMT transgene was expressed in immature seeds, but not in leaves, confirming that the vicillin promoter controlled the expression of γ -TMT transgene in transgenic perilla in a seed-specific manner (Fig. 2).

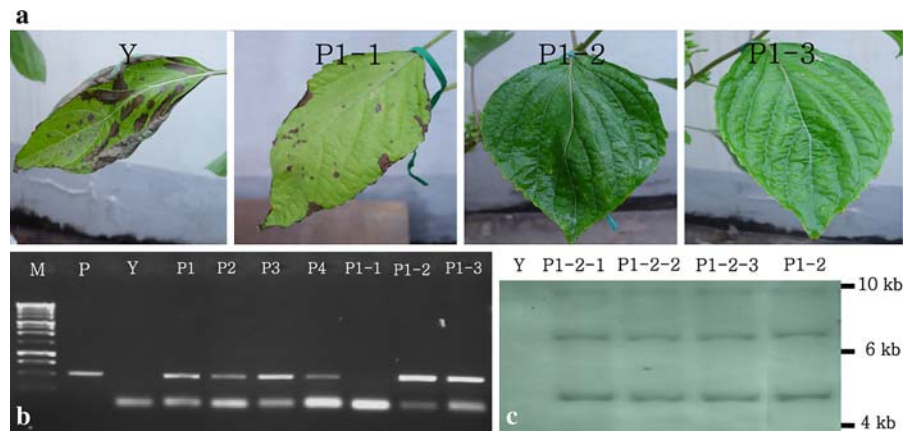


Fig. 1 Confirmation of transgene inheritance and segregation in perilla. **(a)** Inheritance and segregation of herbicide resistance transgene among T_1 progeny plants derived from the transgenic T_0 plant (P1). 0.5% BASTA was applied onto a leaf at seed mature stage. Y, Yeupsil untransformed control; P1-1, a T_1 plant of P1 without transgene due to segregation; P1-2 and P1-3, T_1 progenies of P1 carrying transgene. **(b)** PCR-amplification of transformed γ -TMT gene. M, molecular size

marker P, plasmid (pBK I) as a positive control; Y, Yeupsil as a negative control; P1–P4, transgenic T_0 plants recovered from different transformation events; P1-1, a T_1 progeny of P1 that does not carry the transgene due to segregation; P1-2 and P1-3, T_1 progenies of P1 carrying the transgene. **(c)** Southern blot analysis of T_2 plants (P1-2-1, P1-2-2, and P1-2-3) derived from the same T_1 plant (P1-2). Y, Yeupsil as a negative control; P1-2, T_1 parent of P1-2-1, 2 and 3

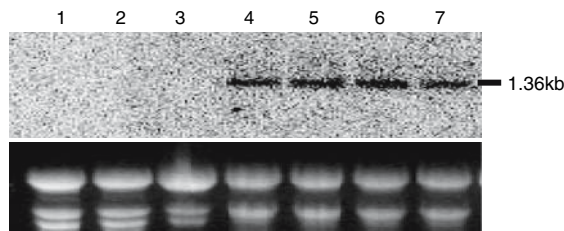


Fig. 2 Northern blot analysis of transformed γ -TMT gene transcript. Upper panel: Northern blot. lane 1, Yeupsil leaf; lane 2, T_0 plant (P2 leaf); lane 3, Yeupsil (immature seed) 10 days after flowering; lane 4–7, Immature seeds of P1-2, P1-3, P2-1, and P2-2, respectively, 10 days after flowering. Lower panel: ethidium-bromide gel staining of RNA samples as loading control

Tocopherol content, fatty acid composition and total lipid content in transgenic perilla seeds

The concentration and composition of tocopherol and the HPLC chromatogram for tocopherols isolated from perilla seeds are presented in Table 1 and Fig. 3. As shown in Table 1, α -tocopherol content increased dramatically in transgenic perilla seeds relative to the control. In the seeds of Yeupsil cultivar, α -tocopherol and γ -tocopherol contents ranged 0.9–11.4 mg/100 g and 199.4–272.8 mg/100 g, respectively depending on the growing season; that is, most of the tocopherol in untransformed

perilla seeds existed as γ -tocopherol. Whereas, in the seeds of three T_0 plants (P2, P3, and P4), α -tocopherol and γ -tocopherol contents were 153.2–200.4 mg/100 g, and 37.1–59.6 mg/100 g, respectively, showing a significant increase of α -tocopherol due to the conversion from γ to α form. Among the six T_1 plants (P1-1, P1-2, P1-3, P2-1, P2-2, and P2-3) derived from two T_0 lines (P-1 and P-2), P1-1 did not show any significant change in tocopherol content and composition as compared with Yeupsil. Clearly, this progeny plant did not carry the γ -TMT transgene due to the progeny segregation (Figs. 1, 3, and Table 1). However, in the seeds of other T_1 plants with transformed γ -TMT, the high α -tocopherol content in seeds was inherited. In transgenic T_2 seeds, the α -tocopherol content was also significantly higher than in control seeds, confirming that the γ -TMT gene was not only transmitted to and but also expressed well in the T_2 progeny plants, leading to high α -tocopherol content phenotypes. Although the total tocopherol content in the T_2 seeds was lower than T_1 seeds, this was probably due to the different growth condition year after year, the ratio of α/γ -tocopherol was maintained high (Table 1).

As for the fatty acid composition and total lipid content, neither fatty acid composition nor total lipid content in the transgenic perilla seeds exhibited

Table 1 Tocopherol composition and content in the seeds of the T₀, T₁, and T₂ transgenic lines

Transgenic lines	Tocopherols (mg/100 g)			α/γ^a
	α	γ	Total	
Yeupsil	11.4	233.1	244.5	0.05
T ₀ (P2)	192.5	37.1	229.6	5.19
T ₀ (P3)	153.2	59.6	212.8	2.57
T ₀ (P4)	200.4	48.0	248.4	4.17
Mean ^b	182.0 ± 25.3	48.2 ± 11.3	230.3 ± 17.8	3.98 ± 1.32
Yeupsil	0.9	272.8	273.7	0.00
T ₁ (P1-1)	12.2	243.3	255.3	0.05
T ₁ (P1-2)	179.0	83.1	262.1	2.15
T ₁ (P1-3)	221.0	44.8	265.8	4.93
T ₁ (P2-1)	161.2	38.4	199.6	4.20
T ₁ (P2-2)	139.4	39.1	178.5	3.57
T ₁ (P2-3)	160.3	65.5	225.7	2.45
Mean ^c	153.6 ± 12.3	47.7 ± 15.5	201.3 ± 23.7	3.41 ± 0.89
Yeupsil	5.8	199.4	205.2	0.03
T ₂ ^d	131.6 ± 18.9	43.6 ± 13.8	175.2 ± 23.6	3.50 ± 1.9

^a The ratio of the α -tocopherol content over γ -tocopherol content

^b Data were the average of three T₀ plants (P2, P3, and P4) ± standard deviation

^c Data were the average of five T₁ plants (P1-2, P1-3, P2-1, P2-2 and P2-3) ± standard deviation

^d data were the averages of six T₂ progeny plants (P1-2-1, P1-2-2, P1-2-3, P1-3-1, P1-3-2, and P1-3-3) ± standard deviation

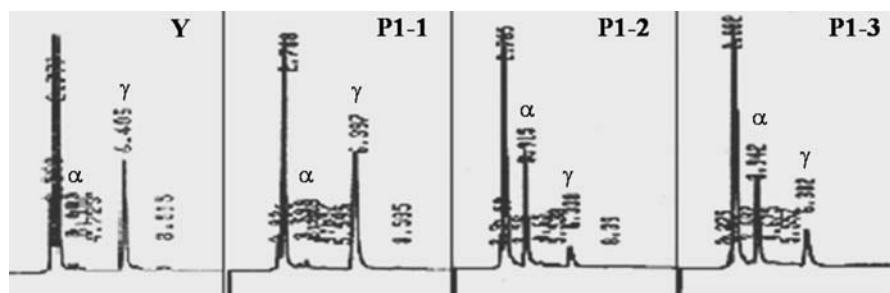


Fig. 3 HPLC Chromatograms of the tocopherols in the seeds of Yeupsil, and three T₁ plants transformed with γ -TMT gene. Y, Yeupsil; P1-1, progeny plant that does not carry γ -TMT transgene due to segregation; P1-2 and P1-3, progeny plants

that carry γ -TMT transgene. Note: α indicates α -tocopherol and γ indicates γ -tocopherol. Numbers above each peak indicate retention time

significant differences from the untransformed control Yeupsil (Table 2).

Discussion

Genetic transformation has been used as an efficient means of improving the nutritional quality of plants (Hirschberg 1999; Cho et al. 2005; Yu et al. 2000; Bettina and Richard 2005) and conferring resistance of plants to biotic or abiotic stress (Singh et al. 2005; LeDuc et al. 2004; Shou et al. 2004). Our results in this study further illustrate the merit of the genetic engineering approach in enhancing crop nutritional

value. This is the first report on the seed specific expression of endogenous perilla γ -TMT gene in perilla itself.

The principal function of α -tocopherol is as an antioxidant in plant cell, one that can benefit human or animal health. Here we focused on the nutritional effect of the α -tocopherol for human health. That is, α -tocopherol possesses the highest vitamin E activity among the four different tocopherol forms (Weiser et al. 1996; Eitenmiller 1997). It is also known that only α -tocopherol is retained and distributed throughout the body during digestion (Shintani and DellaPenna 1998).

In perilla seeds, the major tocopherol component is γ -tocopherol, as in other commercial oil crops such as

Table 2 Fatty acid composition and total lipid content of T₀, T₁, and T₂ transgenic seeds

Transgenic lines	Fatty acid (%)					Total lipids (%)
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	
Yeupsil	7.2	2.6	13.2	12.1	64.7	47.6
T0 (P2)	8.3	3.0	11.6	15.2	62.0	48.1
T0 (P3)	9.0	2.8	13.1	14.8	60.4	47.9
T0 (P4)	8.0	2.9	16.2	13.0	59.9	43.9
Mean ^a	8.4 ± 0.5	2.9 ± 0.1	13.6 ± 2.4	14.3 ± 1.2	60.8 ± 1.1	46.6 ± 2.4
Yeupsil	7.7	2.7	13.1	15.5	61.4	48.7
T1 (P1-1)	8.5	3.2	14.6	13.5	60.2	48.3
T1 (P1-2)	8.9	2.9	13.8	14.2	60.2	44.4
T1 (P1-3)	10.1	5.3	11.6	14.1	58.8	46.5
T1 (P2-1)	7.8	2.9	14.3	16.6	58.4	50.8
T1 (P2-2)	8.2	3.1	13.3	14.1	61.4	50.4
T1 (P2-3)	9.4	2.9	14.8	17.9	56.5	50.8
Mean ^b	8.9 ± 0.9	3.4 ± 1.1	13.6 ± 1.2	15.4 ± 1.8	59.1 ± 1.9	48.6 ± 3.0
Yeupsil	7.5	2.6	13.2	13.6	61.6	48.4
T2 ^c	9.0 ± 1.7	3.2 ± 0.6	13.6 ± 1.9	16.8 ± 0.5	57.4 ± 3.4	50.0 ± 0.8

^a Data were the average of three T₀ plants (P2, P3, and P4) ± standard deviation

^b Data were the average of five T₁ plants (P1-2, P1-3, P2-1, P2-2 and P2-3) ± standard deviation

^c Data were the averages of six T₂ progeny plants (P1-2-1, P1-2-2, P1-2-3, P1-3-1, P1-3-2, and P1-3-3) ± standard deviation

corn and soybean (Grusak 1999). In order to improve the nutritional quality of perilla seeds, we tried to demonstrate the γ -TMT gene expressed faithfully in the seeds by using a tissue-specific promoter. We also tried to demonstrate that the γ -TMT transgene was faithfully transmitted to and expressed in the progeny. To achieve γ -TMT gene expression in a seed-specific manner, the vicillin promoter isolated from pea (Higgins et al. 1988) was used. The vicillin promoter apparently functions faithfully in perilla seed as well. However, it is not clear if this promoter can drive transgene expression in tissues other than seeds, since we have not assessed its expression in tissues other than seeds and leaves.

HPLC analyses of tocopherol in the seeds revealed that most of the γ -tocopherol in the seeds of T₀ plants was converted to α -tocopherol (Table 1, Fig. 3). This is apparently the result of efficient conversion from γ -tocopherol. In addition, this desirable trait was inherited to the T₁ and T₂ progeny, since the transgene was stably transmitted to subsequent generations (Fig. 1). In fact, both the high α -tocopherol content in the transgenic seeds and the transformed γ -TMT gene have been stably transmitted to the T₃,

T₄, and T₅ generation (Data not shown). The analysis of total lipid content and fatty acid composition showed that there was no significant difference between untransformed control and transgenic perilla lines. This suggested that modulation of pathways in vitamin E synthesis did not significantly impact seed total lipid contents and fatty acid compositions of the seeds. The resultant transgenic perilla with high α -tocopherol content is now being used as a breeding material for crosses with other elite Korean perilla varieties (Data not shown). This further illustrates the economical value of the genetic engineering approach.

Acknowledgment We thank Dr. Seth D. Findley (University of Missouri-Columbia) for critical review of this manuscript.

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